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UTILIZATION OF STABLE CARBON ISOTOPE IN THE VERIFICATION OF BIOREMEDIATION OF CHLORINATED HYDROCARBONS

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13. ABSTRACT (Maximum 200 words)
The overall goal of this work was to test the feasibility of using stable carbon isotope analysis to verify *in situ* biodegradation of hydrocarbons and chlorinated ethenes under aerobic conditions. Specific objectives were to 1) determine d13C values for TCE and the various compounds to be used as co-substrates for TCE degradation; 2) evaluate effects of aerobic biodegradation of co-substrate compounds and TCE on gross fractionation of carbon isotopes as measured in the resulting CO2; 3) test the carbon isotope method for its ability to differentiate the source of the CO2 resulting from aerobic biodegradation; and 4) use samples from an appropriately contaminated bioventing site. Different degrees of fractionation were measured in both pure and mixed cultures for the different compounds tested in this study. Fractionation for toluene was slightly more negative than the starting compound. For JP-4, the fractionation of JP-4 resulted in d13C values much more negative than the starting compound. Fractionation for TCE results in d13C values more positive, indicating the CO2 had a higher percentage of 13C than the source compound. A mixture of toluene and TCE had a d13C value intermediate between the two. These results indicate, with the d13C values for these co-substrates and chlorinated ethenes sufficiently different, the degradation of one or the other can be determined. When both are being degraded an intermediate value can be obtained.

14. SUBJECT TERMS
Stable carbon isotope analysis, d13C values, TCE, JP-4 jet fuel, bioventing, fractionation, aerobic biodegradation, chloroethenes

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PREFACE

This report was prepared by Envirogen, Inc., 4100 Quakerbridge Road, Lawrenceville NJ 08648, for the Armstrong Laboratory Environics Directorate (AL/EQ), Suite 2, 139 Barnes Drive, Tyndall Air Force Base, Florida 32403-5323.

The overall goal of this work was to test the feasibility of using stable carbon isotope analysis to verify *in situ* biodegradation of hydrocarbons and chlorinated ethenes under aerobic conditions. Specific objectives were to 1) determine $d^{13}C$ values for TCE and the various compounds to be used as co-substrates for TCE degradation; 2) evaluate effects of aerobic biodegradation of co-substrate compounds and TCE on gross fractionation of carbon isotopes as measured in the resulting CO_2; 3) test the carbon isotope method for its ability to differentiate the source of the CO_2 resulting from aerobic biodegradation; and 4) use samples from an appropriately contaminated bioventing site. Different degrees of fractionation were measured in both pure and mixed cultures for the different compounds tested in this study. Fractionation for toluene was slightly more negative than the starting compound. For JP-4, the fractionation of JP-4 resulted in $d^{13}C$ values much more negative than the starting compound. Fractionation for TCE results in $d^{13}C$ values more positive, indicating the CO_2 had a higher percentage of ^{13}C than the source compound. A mixture of toluene and TCE had a $d^{13}C$ value intermediate between the two. These results indicate, with the $d^{13}C$ values for these co-substrates and chlorinated ethenes sufficiently different, the degradation of one or the other can be determined. When both are being degraded an intermediate value can be obtained.

The work was performed between July and December of 1995. The AFRL/MLQE project officer was Catherine M. Vogel. This is a Phase I Small Business Innovative research (SBIR) Report.

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EXECUTIVE SUMMARY

A. OBJECTIVES

The overall goal of this work was to test the feasibility of using stable carbon isotope analysis to verify *in situ* biodegradation of hydrocarbons and chlorinated ethenes under aerobic conditions. Specific objectives were:

1. To determine the $\delta^{13}C$ values for TCE and the various compounds to be used as cosubstrates for TCE degradation. $\delta^{13}C$ values are the ratio of $^{13}C/^{12}C$ relative to the PeeDee belamnite (PDB) standard.

2. To evaluate the effects of aerobic biodegradation of cosubstrate compounds and TCE on gross fractionation of carbon isotopes as measured in the resulting carbon dioxide (CO_2).

3. To test the carbon isotope method for its ability to differentiate the source of the CO_2 resulting from aerobic biodegradation of two carbon sources simultaneously (i.e. the cosubstrate and the chlorinated hydrocarbon).

4. To use samples from an appropriately contaminated bioventing site to do a small-scale field test of this technology.

B. BACKGROUND

Recently, *in situ* bioremediation systems, such as bioventing and biosparging, have been applied to numerous sites to enhance existing subsurface environments for petroleum hydrocarbon degradation. In an effort to monitor the effectiveness of these systems, several approaches have been explored to provide evidence of biological degradation. Of these, only methods involving detailed mass balance equations utilizing measurements of changes of the petroleum

hydrocarbons and their metabolic intermediates can provide direct evidence of biological degradation of the petroleum hydrocarbons. However, due to the non-homogenous distribution of petroleum hydrocarbons and their metabolic intermediates in most subsurface environments, it is generally impractical to perform a mass balance.

One method currently being considered as a possible alternative for verifying the occurrence of biological degradation is the use of stable carbon isotopes. This method involves the examination of carbon-containing respiration products from biodegradation to determine the source of the carbon. The stable carbon isotope method is employed by comparing the stable carbon isotope composition of the CO_2 with the potential source material. The isotopic composition by convention is referenced to the PeeDee Belemnite (PDB) standard and is reported as the $\delta^{13}C$ of the sample in parts per thousand (per mil) deviation from the PDB standard. For example, a negative $\delta^{13}C$ value indicates that the sample is depleted in carbon-13 relative to the PDB standard. Due to differences such as mass and nuclear spin of the carbon-12 and carbon-13 atoms, the carbon isotopes behave slightly differently when undergoing reactions (Galimov, 1985). The resulting change in isotopic composition of the reaction products relative to the reactants is referred to as fractionation. If the fractionation associated with biological degradation processes can be estimated, then the isotopic composition of the original carbon substrates can be deduced.

Unlike petroleum hydrocarbons, which serve as primary substrates for degradative bacteria, chlorinated ethenes such as trichloroethylene (TCE) are degraded cometabolically under aerobic conditions. Specific primary substrates are required for induction of the appropriate enzyme systems. Hydrocarbons, often found as co-contaminants with chlorinated ethenes, can induce indigenous bacteria to degrade a number of chlorinated ethenes. It is hypothesized that if the primary substrate and the chlorinated solvent $\delta^{13}C$ and the fractionation associated with their degradation is known, the relative proportion of cosubstrate to chlorinated substrate degradation can be deduced (i.e. the CO_2 source can be determined).

C. SCOPE

This report details work performed to determine if the stable carbon isotope method enables source differentiation of CO_2 resulting from the biodegradation of two carbon compounds simultaneously, in this case, a cosubstrate and a chlorinated hydrocarbon.

This Phase I feasibility study consisted of:

Task 1: measurement of stable carbon isotope ratios of TCE and compounds which act as cosubstrates and inducers of TCE degradative activity.

Task 2: microcosm studies of pure cultures of well-characterized TCE-degrading microorganisms to determine whether fractionation occurs when these compounds are aerobically degraded.

Task 3: microcosm studies with pure and mixed TCE-degrading cultures to determine isotope ratios obtained when both TCE and the cosubstrate compounds are present.

Task 4: analysis of samples from an appropriate bioventing site to determine whether *in situ* biodegradation of TCE and hydrocarbons can be determined by carbon isotope analysis from soil gas samples.

D. METHODOLOGY

During this project ENVIROGEN employed GeoChron Laboratories (Krueger Enterprises) of Cambridge, Massachusetts for stable carbon isotope analysis. GeoChron Laboratories analyzed the initial organic compounds and the final product as CO_2 to determine the respective $\delta^{13}C$ values at the beginning and end of the experiments. In addition to GeoChron Laboratories, samples were analyzed by Professor Teofilo Abrajano of the Department of Earth Sciences at Memorial University of New Foundland in St. Johnís, New Foundland Canada.

For this Phase I work, ENVIROGEN made use of its collection of TCE-degrading bacteria. Many of the bacterial cultures were obtained from contaminated sites and have been enriched

within the laboratory on a single carbon source. These bacterial cultures were used in microcosm studies designed to determine whether the stable carbon isotope ratio of CO_2 resulting from degradation of chlorinated ethenes and hydrocarbons would reflect the degradation of one or both of these compounds.

E. TEST DESCRIPTION

Stable carbon isotope analysis was performed on CO_2 samples collected from both closed- system laboratory microcosm studies and soil gas samples collected in the field. In both cases, the contaminants of concern included TCE and a variety of cosubstrates including petroleum hydrocarbons. Microcosm systems were operated in both batch mode, with the contaminant compounds added at the beginning of the experiment, and in a continuous mode, with the contaminants supplied as vapors throughout the experiment. These continuous mode microcosms were designed to more closely mimic field conditions of degradation.

Field samples were taken at a former UnoCal Chemicals facility in Denver Colorado. The site soils and groundwater are contaminated by a mixture of chlorinated ethanes (PCE, TCE, DCE and VC) as well as petroleum hydrocarbons (toluene and benzene). The site consultants, Camp Dresser & McKee, Inc., provided the samples used in this study. Field samples were analyzed for both the stable carbon isotope ratios of the contaminants in the groundwater and the resulting CO_2 in soil gas samples. Because there was more than one contaminant at this site, the field samples were analyzed by first separating the components of the mixture and then analyzing the stable carbon isotope ratio of each component. In addition to the field samples, groundwater from the site was used in microcosm studies to determine whether field results could be duplicated in the laboratory microcosm system.

F. RESULTS

The $\delta^{13}C$ values for the target contaminants were within the range of reported values. Laboratory microcosm studies demonstrated carbon isotope ratios in CO_2 resulting from degradation did not vary significantly for different bacterial strains with different enzyme systems. Microcosm studies also determined stable isotope ratios in CO_2 evolved as a result of degradation by a mixture of organisms; the results of these studies correlated well with the pure culture results. In the cases where there was a lag period before degradation, $\delta^{13}C$ values for the CO_2 in microcosms containing degradative bacteria and substrate did not vary significantly until degradation of the compound was initiated.

The lack of correlation between the location of soil vapor and groundwater samples and by the number of contaminants present at the Denver site made the field results difficult to interpret. However, at locations where both soil vapor and water samples were taken in close proximity, the $\delta^{13}C$ values obtained in the field were very similar to the $\delta^{13}C$ values obtained in microcosm studies. In addition, in site samples enriched for toluene-degrading microorganisms, the degradation of single and multiple substrates yielded $\delta^{13}C$ values similar to those in our microcosm studies with pure cultures. This indicates the microcosm system developed in this Phase I work is predictive of $\delta^{13}C$ values obtained in the field. The advantage to the microcosm studies is degradation can be directly measured in these closed systems and correlated to degradation taking place in the field by the isotope ratio of the evolved CO_2.

Different degrees of fractionation were measured in both pure and mixed cultures for the different compounds tested in this study. Fractionation for toluene was slightly more negative than the starting compound. For JP-4, the fractionation or differences in mineralization of the various components of JP-4 resulted in $\delta^{13}C$ values much more negative than the starting compound. More negative values for the $\delta^{13}C$ of the CO_2 indicate the CO_2 was lighter or had a higher percentage of ^{12}C than the starting compound. Fractionation for TCE results in $\delta^{13}C$

values more positive, indicating the CO_2 had a higher percentage of ^{13}C than the source compound. A mixture of toluene and TCE had a $\delta^{13}C$ value intermediate between the two. These results indicate, with the $\delta^{13}C$ values for these cosubstrates and chlorinated ethenes sufficiently different, the degradation of one or the other can be determined. When both are being degraded an intermediate value can be obtained.

The effect of differential degradation of mixed compound substrates such a JP-4 needs to be separated from fractionation effects. This can be accomplished by continuing testing residual substrates throughout the course of microcosm studies. This effect can then be separated from actual fractionation effect.

G. CONCLUSIONS

There were considerable technical hurdles to overcome in evaluating this type of system in laboratory microcosm studies. The microcosm system developed in this Phase I work will be useful in future studies for evaluating stable isotope ratio analysis for other contaminants and bioremediation systems. The microcosm system developed in this work, simulates field conditions by maintaining a constant supply of the contaminants to the microorganism. Carbon isotope ratios, as a monitoring technology, will be easier to evaluate in the field where the contaminant supply and the metabolic state of the organisms are both fairly constant. It is hypothesized that some of the variability observed in the microcosm studies are due to the 'batch' nature of the microcosm studies and will not be present in field samples. Field samples will also have less variability due to the averaging effect of a community of microorganisms rather than a single strain.

This study has also shown stable isotope analysis may also be useful in identifying multiple sources of contamination. This is very important for commercial clients who do not want to retain liability for contaminants that have migrated onto their site. In addition to providing an additional tool to demonstrate intrinsic bioremediation, future research would determine whether

stable isotope analysis can be used to optimize a cometabolic bioventing/biosparging process so that remediation is accelerated and endpoints are easily determined for termination of a system and site closure.

H. RECOMMENDATIONS

Although our laboratory results indicated that stable carbon isotope ratio analysis can be used to determine when mixed substrates are being degraded, the fractionation of stable carbon in CO_2 resulting from the biodegradation of organic substrates such as JP-4 and TCE under field conditions is poorly understood. Current data available in the literature (i.e., Aggarwal and Hinchee, 1991; Suchomel, et.al., 1990; Neuber, et.al, 1991; Zyakun, 1992; Van de Velde, et.al., 1995) is inadequate for evaluating fractionation potentials under field conditions. Likewise, as of this date, no field studies have been conducted using stable chlorine isotope analysis to verify biodegradation of chlorinated organic compounds. These data should be generated through the construction of controlled field test plots, systematic field and laboratory testing, and the application of both carbon and chlorine stable isotope analyses.

A laboratory study, based upon the results from this Phase I work, should be designed that is closely tied to a field sampling program. The objective of this approach would be to verify microcosm studies can be used to predict the isotope ratios that would be expected from degradation of one or more contaminants.

ENVIROGEN recommends Phase II studies be performed in a field-scale ëclosedí plot in order to demonstrate this system at the field scale in a very well-defined system. A bioventing system should be installed at a site contaminated by TCE and JP-4 where the vadose zone can be isolated in a 50' x 50' plot. Bioventing with both air and pure oxygen will enable us to determine the contribution of the CO_2 in the venting air.

In addition to stable carbon isotopes in soil gas samples, analysis of stable chlorine isotopes in groundwater samples may provide additional evidence for degradation of chlorinated ethenes and help to close the mass balance on the degradation without extensive sampling.

Comparing the results obtained in a well-controlled laboratory and field system to stable isotope analysis at other bioventing sites will enable ENVIROGEN to develop a protocol for monitoring bioventing and biosparging sites. These protocols will enable the user to demonstrate degradation of both cosubstrate and chlorinated ethenes with inexpensive soil gas and groundwater sampling. These protocols may also provide users with parameters which can be used to monitor the progress of bioventing/biosparging processes and enable efficient termination of these systems at scientifically determined endpoints.

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TABLE OF CONTENTS

TABLE OF CONTENTS (Cont'd)

LIST OF TABLES

SECTION I

INTRODUCTION

There is a tremendous need for *in situ* technologies for remediating contaminated soils and groundwaters in the United States. A large percentage of the nation's pollution problems exist in soils and aquifers where *ex situ* treatment is generally expensive and often technically impossible to accomplish. *In situ* technologies, which can cost-effectively destroy contaminants in place rather than transferring them to another media, thereby decreasing the risk of human exposure, are preferred. *In situ* bioremediation, where applicable, is such a preferred technology.

Cometabolic bioventing/biosparging is an innovative, promising technique which may be used to promote *in situ* biodegradation of some of the more recalcitrant chlorinated ethenes when they are co-contaminants with petroleum hydrocarbons. The problem becomes one of monitoring this process to insure the addition of oxygen to the subsurface is promoting the degradation of both the petroleum and the solvents. Research is needed to better understand the effectiveness of cometabolic degradation and to develop the means to monitor this *in situ* biodegradation. It may be possible to optimize the operation of a cometabolic bioventing system using inexpensive stable isotope analysis for adjusting the parameters to gain optimal degradation of both contaminants.

A. OBJECTIVES

The hypotheses tested in this project were: 1) fractionation coefficients can be determined which describe the gross fractionation occurring when aerobic microorganisms metabolize hydrocarbons; and 2) the fractionation coefficients will allow evaluation of multiple sources of metabolic end products by the stable carbon isotope method. It is the objective of this study to determine whether stable carbon isotope analysis can be used to determine whether more than

one contaminant is being aerobically degraded, specifically chlorinated ethenes and compounds which serve as cosubstrates for degradation of petroleum hydrocarbons.

B. BACKGROUND

Difficulties associated with sampling a heterogeneous field site complicate the task of quantitatively demonstrating bioremediation as the mechanism of contaminant removal. Even intense sampling can show significant error with respect to system mass balance. The task of proving a technology has destroyed contamination *in situ*, and closing a mass balance on those reactions in a "three dimensional mass" of the subsurface, has received considerable attention (Fredrickson et al., 1993). This research was designed to refine and utilize this approach to better understand and control the dual effects of stimulating the degradation of two contaminants, one of which can induce the degradation of the other. It is expected this analysis of stable isotope ratios developed as a monitoring technology will be equally effective in monitoring the progress of *in situ* cometabolic bioventing and biosparging for contaminants in soil and groundwater.

The U.S. Air Force, in collaboration with the U.S. EPA, has developed protocols for bioventing aerobically degradable contaminants in the vadose zone. The Air Force is also supporting considerable field research in the development of air sparging and biosparging technologies for *in situ* remediation of contaminants in the saturated zone. These systems are designed to accelerate biological degradation by oxygenating the subsurface environment. Increased oxygen availability enables the indigenous aerobic microbes to metabolize the petroleum hydrocarbons. At some sites, investigations have shown degradation is occurring naturally (i.e., natural attenuation or passive bioremediation). Researchers have shown, under laboratory conditions, aerobic microbes collected from hydrocarbon release sites are often capable of completely mineralizing many hydrocarbons to CO_2 and water (Atlas, 1991).

The effectiveness of aerobic degradation technologies, however, is very difficult to assess without a very extensive and expensive sampling regime. An *in situ* respiration test, developed to provide an initial assessment of the feasibility of bioventing for contaminated soil, can be used throughout a bioventing program to assess the rate of oxygen consumption and therefore degradation rate (Hinchee, et al 1992). This technology, however, cannot distinguish between degradation of more than one compound. The objective of this research was to develop a stable isotope technology utilizing an inexpensive sampling and analysis protocol to determine the degradation of more than one contaminant.

Very little is known about the biological degradation of chlorinated ethenes under bioventing conditions. Unlike petroleum hydrocarbons which serve as primary substrates for degradative bacteria, chlorinated ethenes, such as trichloroethylene (TCE), are degraded cometabolically. Specific primary substrates are required for induction of the appropriate enzyme systems. Primary substrates, such as toluene, propane, and butane, which can be delivered as vapors in air, can induce indigenous bacteria to degrade a number of chlorinated ethenes (Kampbell and Wilson, 1994). Natural gas, which is a mixture of hydrocarbons, added to a soil column in an air stream resulted in removal of >95% of added TCE during a two day residence time (Wilson and Wilson, 1985).

In addition to the potential for adding cometabolites in vapor during the bioventing process, hydrocarbons are often found as co-contaminants with chlorinated ethenes. There is a wide range of hydrocarbons which act as cometabolites for TCE degradation (McClay et al., 1995; Wilson and Wilson, 1985; Ensley, 1991). Soils acclimated to degrading JP-4 and aviation gasoline have been shown to be very effective in removal of both TCE and vinyl chloride (VC) vapors (Kampbell and Wilson, 1994). In addition, the presence of TCE in soil up to at least 13 ppm was shown not to effect the rate of gasoline biodegradation in soil microcosms (Wilson and Wilson, 1985).

Indigenous soil microorganisms have been shown to readily degrade TCE to CO_2 in the presence of high concentrations of toluene added to soil, with TCE degradation continuing at a slower rate after all the toluene had been removed (Fan and Skow, 1993). This microcosm work and an *in situ* field demonstration of TCE degradation by indigenous microbial populations in the presence of phenol (Hopkins et al., 1993), support pure culture research on aromatic TCE degrading microorganisms (Ensley, 1991). In addition to the well-known inducers toluene, phenol and methane, scientists have identified a number of other hydrocarbons which induce TCE degradative activity. These hydrocarbons are commonly found as co-contaminants of chlorinated ethenes. TCE and cis-DCE have also been demonstrated to induce TCE degradation in *Pseudomonas mendocina* KR1 and strain *Pseudomonas sp.* ENVPC5 (McClay et al., 1995).

Since hydrocarbons serve as a primary substrate and can support the growth of a number of indigenous microorganisms, often the only additional substrate or electron acceptor needed to stimulate biodegradation is oxygen. In the vadose zone, the addition of oxygen by bioventing has been demonstrated to be an effective and economical method of remediating petroleum hydrocarbon contamination. Bioventing is viewed as an improvement of soil vapor extraction (SVE) technology which is used to volatilize contaminants to remove them from the vadose zone. When applicable, bioventing is more desirable than SVE because it enhances biodegradation which is a destruction rather than a removal technology. Chlorinated ethenes, however, do not serve as a primary substrate for bacteria and usually require the addition of an inducer or cometabolic substrate before degradation is observed. Fortunately, components of a number of petroleum hydrocarbon mixtures act as inducers for degradation of chlorinated ethenes like TCE. Cometabolic TCE degradation has been demonstrated at ENVIROGEN with toluene, octane, hexane, and pentane all commonly found as components of petroleum mixtures, including JP-4 (McClay et al., 1995).

Several techniques have been used to provide evidence of biological degradation under field conditions. These approaches have included measurements over time of changes in the concentration of contaminants, number of contaminant specific degradative microorganisms, ratio of rapidly degraded hydrocarbons to slowly degraded hydrocarbons, metabolic intermediates, oxygen uptake and CO_2 generation (Aggerwal and Hinchee, 1991). The bioventing protocol developed by the U.S. Air Force for biodegradation of petroleum hydrocarbons uses *in situ* respirometry. This method measures oxygen uptake and CO_2 generation as a means of determining whether microbial activity is occurring, whether microbial activity is oxygen limited, and, in some cases, to calculate biodegradation rates (Hinchee et al., 1992; Miller and Vogel, 1994). Of these approaches, only detailed mass balance calculations utilizing measurements of changes in the concentration of contaminants and metabolic intermediates can provide direct proof of biological degradation. Unfortunately, because of the heterogeneous distribution of contaminants and their metabolic intermediates, coupled with multiple sources and sinks of metabolites in the environment, numerous measurements are needed to calculate a mass balance and verify biological degradation. The collection and analysis of these samples is very expensive and time-consuming. With the significant growth in the use of bioremediation, new cost effective and reliable methods must be developed to verify, monitor, and model biological degradation in the field.

One method which has received some attention as an alternative approach for verifying biological degradation of organic carbon compounds in the field is the analysis of stable carbon isotope ratios (Aggerwal and Hinchee, 1991; Suchomel et al., 1990; Neuber et al., 1991; Zyakun, 1992; Van de Velde et al., 1995). With this method, carbon-containing end products from biodegradation are examined to determine the initial source of the carbon in the end products. The carbon-containing end product from the aerobic biological mineralization of hydrocarbons is typically CO_2. Although changes in the CO_2 concentrations have been utilized in the past to demonstrate biodegradation, several other sources of CO_2 such as the atmosphere, biological degradation of organic carbon compounds other than the hydrocarbon of interest, plant root

respiration, and calcite precipitation can interfere with this method unless the CO_2 from these sources can be discerned. Through the measurement of stable carbon isotope ratios, it is possible that the amount of CO_2 contributed from multiple sources, including the biodegradation of the hydrocarbon of interest, can be determined.

Carbon is comprised primarily of two stable isotopes, carbon-12 and carbon-13, in a ratio of approximately 98.89% carbon-12 to 1.11% carbon-13. The stable carbon isotope method is employed by examining the stable carbon isotope composition of the biodegradation end products such as CO_2 or other carbon-containing end products. This CO_2 isotope composition is then compared to possible source substrates. Because of differences such as mass and nuclear spin of the carbon-12 and carbon-13 atoms, the carbon isotopes behave slightly differently when undergoing chemical and biological reactions (Galimov, 1985). As a result, the isotopic composition of an endproduct (i.e. CO_2) may differ from the isotopic composition of the original reactants (i.e. source substrates). This change in isotopic composition of the products of reactions relative to the reactants is referred to as fractionation and must be accounted for in the comparison of the end product with possible source substrates. If the fractionation associated with the biological degradation process can be estimated, then the isotopic composition of the original source substrates can be deduced. The isotopic compositions of the possible original source substrates are then compared to the deduced original substrates to find the actual source substrate for the CO_2. Other stable isotopes, including chlorine, could also be used to determine sources (Van Warmerdam et al., 1995).

Recent advancements in analytical chemistry have opened the possibilities for the application of stable chlorine isotope analysis in environmental and groundwater applications. Chlorine is composed of two primary stable isotopes, C-35 and C-37. The overall range of chlorine isotope ratios previously identified in natural fluids is small, and typically ranges 0.0+/-1.0 per mil relative to ocean water (personal communication Andrew Magenheim, Ph.D., 1995). In a recent paper, researchers reported that the chlorine isotope ratios for several chlorinated ethenes (i.e.,

TCE, PCE, and TCA) from various manufacturers were widely variable, ranging from -2.9 to +4.1 per mil (van Warmerdam et al., 1995). More recent findings by the same research group have found variations in solvent chlorine isotope ratios ranging from -10.0 to +10.0 (personal communication S.K. Frape, 1996).

Although the laboratory analysis procedures for determining stable chlorine isotope ratios in inorganic and organic forms is currently available, only one field application of the method has been conducted. In this instance, the inorganic chlorine dissolved in groundwater in the vicinity of a TCE plume was sampled and analyzed for the stable chlorine isotope ratio. Inorganic chlorine outside of the TCE plume was shown to have an isotope ratio of +1.0 per mil while the chlorine inside the TCE plume had an isotope ratio of +4.0 per mil which was identical to the isotope ratio of the source compound (personal communication S.K. Frape, 1996).

Because of the limited data regarding the fractionation of carbon and chlorine isotopes by aerobic microorganisms, application of the stable carbon and chlorine isotope methods has been limited. Knowledge of the gross fractionation resulting from the aerobic biodegradation of various carbon and chlorine containing compounds will significantly strengthen this method and promote its broader use at bioremediation sites. It will also allow an evaluation of "natural" biodegradation occurring at a site and potentially validate bioattenuation arguments.

The potential value of the stable isotope method for validating *in situ* bioremediation or bioattenuation is great. If successfully developed, this method will provide the following benefits:

1. Confirmation of biodegradation: Stable isotope analysis can provide strong evidence of complete mineralization of the targeted substrate.

2. Reduced analytical costs: Stable isotope analysis is relatively inexpensive ($30-$65 per sample) compared to other analyses.

3. Fewer analyses: Fewer samples will be required to obtain a statistically representative sample of the soil gas due to less heterogeneity in gas phase samples compared to sludge, soil and water samples.

4. Reduction of sampling costs: Fewer samples and simpler collection procedures for gas samples reduce the amount of time and cost required to collect samples.

C. SCOPE

This report details work performed to determine if the stable carbon isotope method enables source differentiation of CO_2 resulting from the biodegradation of two carbon compounds simultaneously, in this case, a cosubstrate and a chlorinated hydrocarbon

Task 1. Determine $\delta^{13}C$ values for the cosubstrate compounds and TCE.

To determine the $\delta^{13}C$ values for each of the organic compounds used as primary substrates for the microorganisms, and the $\delta^{13}C$ values for TCE which is cometabolically degraded by these microorganisms. Samples of each of the organic compounds, before any biological treatment, were analyzed by Geochron Laboratories using one of two VG Isogas stable isotope ratio mass spectrometers. The pure compounds were converted to CO_2 by high temperature combustion in the presence of pure oxygen, CuO or both. The CO_2 was then separated cryogenically from any other components in the vapor phase prior to isotopic analysis.

Task 2. Evaluate the effects of aerobic biodegradation of cosubstrate compounds and TCE on gross fractionation as measured in the resulting CO_2.

Experiments were performed to measure the effects of aerobic biodegradation of a variety of carbon sources on the $^{13}C/^{12}C$ ratios of the resultants CO_2. Source compounds of known $^{13}C/^{12}C$ (determined in Task 1) were degraded by pure or mixed cultures of degradative bacteria and the $^{13}C/^{12}C$ ratio of the resulting CO_2 was measured. Comparison of the initial ratios (source

compounds) with the final (product compounds; CO_2) provided the basis for the fractionation values.

Two types of metabolism were represented by the microorganisms and test compounds chosen for this work. Primary metabolism where the test compound is a sole source of carbon and energy for the bacteria, and co-metabolism where the test compound is degraded, but is not a growth substrate or energy source for the bacteria.

Task 3. Test the carbon isotope method for its ability to differentiate the source of the CO_2 resulting from aerobic biodegradation of carbon sources.

Experiments were performed to test the ability of the stable carbon isotope method to determine the source of CO_2 produced during aerobic biodegradation of mixtures of carbon compounds. During Task 2, we determined the effects of different individual source compounds and degradative organisms on the carbon isotope ratios of the CO_2 produced during biodegradation. However, to use this technology for routine monitoring in the field we had to be able to differentiate between CO_2 produced from multiple sources. The multiple sources include degradation of mixtures of natural and contaminant organic compounds by mixtures of degradative organisms. During this task, we analyzed stable isotope ratios of CO_2 produced during the aerobic biodegradation of mixtures of common environmental contaminants. The resultant carbon isotope ratios were compared to ratios generated from the individual compounds to determine if the method can be used to estimate the progress of bioremediation efforts in the field.

In our initial experiments, the following mixtures of chemicals were tested: toluene and TCE; methane and TCE; propane and TCE; TCE and gasoline; and TCE and JP-4 fuel. These test chemicals are either common environmental contaminants occurring as major components of complex mixtures at hydrocarbon contamination sites (toluene and JP-4), or they have the potential to be added as co-substrates (propane and methane) in the vapor stream at a bioventing site.

Task 4. Small-scale field test of this technology at an appropriately contaminated bioventing site.

Bioventing has been applied at numerous contaminated sites across the United States. A site was chosen which contains both TCE and at least one known hydrocarbon inducer of TCE degradative activity as co-contaminants in the vadose zone. Carbon isotope ratios of the CO_2 collected from each site was analyzed, and the results compared to laboratory results. Differences in concentrations of non-degraded hydrocarbons and total organic and inorganic carbon were evaluated to confirm the possible sources of CO_2.

SECTION II

Determination of Isotopic Ratios for Cosubstrate Compounds and TCE

Introduction

The purpose of this task was to acquire baseline isotopic ratio values of target substrates and co-substrates for later comparison to CO_2 liberated during biodegradation of these compounds.

Materials and Methods

One to two drops of each of the cosubstrate compounds were aliquotted into teflon-sealed 2 mL glass serum vials and sent to GeoChron Laboratories. GeoChron Laboratories performed the Stable Carbon Isotope Ratio Analysis (SCIRA) on the samples. Samples from these aliquots were combusted at 850°C in a pure oxygen atmosphere. The liberated gases were recirculated for ten minutes over CuO, also at 850° C, to ensure complete combustion. After cooling, the combustion products were separated cryogenically and the isolated CO_2 volume was measured. The sample was then analyzed using a VG Isogas stable isotope mass spectrometer.

Results and Discussion

Results of $\delta^{13}C$ analysis of the test target compounds are presented in Table 1. CO_2 in laboratory air had a $\delta^{13}C$ of -12 per mil. Isotope ratios for the source compounds and cosubstrates were compared to literature values as a verification of the methodology. The reported ranges for these compounds are wide due to the variety of source compounds from which they were manufactured. Analysis of the isotope ratio for any one source compound, however, will not vary significantly as seen in the duplicate analyses for toluene, chlorobenzene and methane (Table 1). These wide ranges also showed significant overlap of the isotope ratios for the different compounds. Much less overlap exists for the specific source compounds used in this study, however, these values indicate that it may be difficult to distinguish between the isotope ratios of Jet A, chlorobenzene, toluene and JP-4, depending on the exact ratio of the starting

compound. The differences observed in $\delta^{13}C$ values for these starting compounds suggested that the $\delta^{13}C$ values for the CO_2 resulting from their degradation could in some cases be differentiated.

Table 1. Carbon Isotope Analysis Results for Selected Compounds

Substrate	Reported Range (del-C^{13})	Ref.	Laboratory Isotope Ratio (del-C^{13})
Trichloroethene (TCE)	-31.9 to -27.8	1	-31.8
JP-4	-34.0 to -20.0*	2	-25.4
Toluene	-34.0 to -20.0*	2	-27.5 -27.4
Chlorobenzene	not available	-	-27.6 -27.3
Methane	-90.0 to -20.0	3	-41.2 -41.3
Propane	-28.6	4	-29.8
Jet A	-34.0 to -20.0*	2	-27.3
cis-Dichloroethene (cis-DCE)	not available	-	-15.2
trans-Dichloroethene (trans-DCE)	not available	-	-10.0

* reported range is typical for petroleum hydrocarbons

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2. Fuex, A.N. J. Geochem. Explor. 6:139-162, 1977
3. Coleman, D.D., et. al., Petroleum Rep.111, Illinois State Geological Survey, 1977
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SECTION III

Evaluation of the Effects of Aerobic Biodegradation of Cosubstrate Compounds and TCE on Gross Fractionation as Measured in the Resulting CO_2.

Introduction

The purpose of this preliminary work was to establish experimental parameters and protocols for testing the effect of biodegradation on δ13C values of CO_2 resulting from the degradation of several chemicals. Results from these experiments were used to determine the effects of different individual source compounds and degradative organisms on the carbon isotope ratios of the CO_2 produced during biodegradation. In addition, these closed system assays looked at factors such as rates of degradation, CO_2 evolution, and the effects of substrate limitation.

Materials and Methods

1. Cultures and Growth Condition

The pure cultures used in this experiment were: *Pseudomonas mendocina* KR1 containing toluene para monooxygenase (TMO) (Winter et al., 1989); *Burkholdia cepacia* PR1 containing toluene ortho monooxygenase (TOM) (Shields et al., 1989); *Pseudomonas putida* F1 containing toluene dioxygenase (TDO) (Wackett and Gibson, 1988); and *Mycobacterium vaccae* JOB5 containing propane monooxygenase (PMO) (Vestal and Perry, 1969). All cultures (pure and mixed) were grown on Basal Salts Media (BSM) to an optical density (550 nm) greater than 0.5 and supplied toluene vapors, with the exception of JOB5 which was supplied propane vapors.

The mixed cultures designated as Site 1 (Table 7) are enrichments of bacteria from water collected in an oil/water separator operating at the site of a pipeline rupture. The members of this enrichment of bacteria, grown with toluene as a sole source of carbon and energy, were uncharacterized.

2. Experimental Procedure

BSM, used to prepare samples, was purged for at least 25 min. with pure oxygen to remove dissolved CO_2. The BSM was then chilled to 4°C to retard basal metabolism of the test organisms prior to adding the target substrates. This was done to achieve T = 0 levels of CO_2 below detection limits and minimize differences in the amount of CO_2 present in replicate samples. Incubations were performed by adding test organisms suspended in chilled BSM (100 ml; OD_{550}= 0.5 to 2.0) to 160 ml serum vials and adding the target substrates. Serum vials were sealed with Teflon-lined septa and incubated at 30°C. Multiple serum vial microcosms were constructed for each of the target substrates so that several samples could be taken over 24 hours (Tables 3 & 4). The incubations were terminated by the addition of 2.0 ml of 5 M HCl. The samples were then sent to Geochron Laboratories for SCIRA.

3. Data Analysis

Data was analyzed by using the method of Van de Velde et al. (1995) to subtract the contribution of residual and/or basal metabolism-generated CO_2 from the measured isotope values. The equations used were as follows:

For a hypothetical case in which two carbon containing compounds are present in a single container and each undergo an aerobic biological reaction producing CO_2, the $\delta^{13}C$ of the resultant CO_2 can be related to the $\delta^{13}C$ of the two original carbon containing compounds by:

$$\delta^{13}C_{total} = \delta^{13}C_{atmos} \times \{[CO_2]_{atmos}/[CO_2]_{total}\} + \delta^{13}C_2 \times \{[CO_2]_2/[CO_2]_{total}\}$$
$$+ \delta^{13}C_1 \times \{[CO_2]_1/[CO_2]_{total}\}$$ (Van de Velde et al., 1995)

where,

$\delta^{13}C_{atmos}$ = $\delta^{13}C$ of the atmospheric CO_2

$\delta^{13}C_1$ = $\delta^{13}C$ of the CO_2 resulting from the reaction of the first carbon-containing compound

$\delta^{13}C_2$ = $\delta^{13}C$ of the CO_2 resulting from the reaction of the second carbon-containing compound

$\delta^{13}C_{total}$ = $\delta^{13}C$ of the resultant CO_2 after completion of the reactions

$[CO_2]_{atmos}$ =moles of atmospheric CO_2 present in container

$[CO_2]_1$ = moles of CO_2 resulting from the reaction of the first carbon-containing compound

$[CO_2]_2$ = moles of CO_2 resulting from the reaction of the second carbon-containing compound (for example background CO_2 measured in no-substrate controls)

$[CO_2]_{total}$ = total moles of CO_2 after completion of the reactions.

This equation assumes no other sources and/or or sinks of CO_2 are present in the container. The equation can be simplified to:

$$[CO_2]_2 = [CO_2]_{total} \times \{ [\delta^{13}C_{total} - \delta^{13}C_1]/[\delta^{13}C_2 - \delta^{13}C_1] \}$$

which is essentially the same equation utilized by Fry et al. (1978) in studies of grasshopper diets using stable carbon isotope analysis. Since $[CO_2]_{total}$ and $\delta^{13}C_{total}$ are easily measured, $[CO_2]_2$ could be solved if the $\delta^{13}C_1$ and $\delta^{13}C_2$ were known. Once $[CO_2]_2$ is known, $[CO_2]_1$ could be calculated.

The $\delta^{13}C_1$ and $\delta^{13}C_2$ are related to the measurable $\delta^{13}C$ of the original carbon-containing compounds although the amount of fractionation the carbon experienced during the reactions is unknown. This fractionation could be described by:

$\delta^{13}C_1$ = a x $\delta^{13}C$ of the original carbon-containing compound (Parker, 1970)

where, a = fractionation coefficient constant.

The fractionation coefficient (a) as described above assumes fractionation is constant over time, independent of the concentration of the original carbon-containing compound, and does not alter the $\delta^{13}C$ of the original carbon-containing compound. Therefore, in a case where this equation

describes the fractionation, one of the isotopes (i.e. carbon-12 or carbon-13) must be preferentially accumulating in the growing biomass of the degrading bacterial population.

Results and Discussion

Results of representative Task 2 experiments are presented in Tables 2 to 4. These time course studies were designed to examine the effect of substrate concentration on the resulting $\delta^{13}C$ of the CO_2. In preliminary experiments, before it became apparent $\delta^{13}C$ values would have to be corrected for background, measurements of the CO_2 volume were not taken. These uncorrected values indicated fractionation was taking place with the resulting CO_2 being enriched in ^{13}C relative to the substrate (Tables 2, 3 and 4). When these values were corrected, however, the $\delta^{13}C$ values were significantly different from the $\delta^{13}C$ values of the original substrate. For example, when *P. mendocina* KR1 was incubated with 25 ppm toluene (Table 2), toluene degradation did not proceed until after 2 hr. of incubation. Prior to toluene degradation, the sample CO_2 had a carbon isotope ratio of approximately -12, which was representative of CO_2 values measured in the laboratory air. Upon initiation of toluene degradation, the carbon isotope ratio decreased to approximately -18. When adjusted for background CO_2 present in no substrate controls, the carbon isotope ratio of CO_2 produced from toluene was between -22.8 and -27.0. The value of -27.0 at 5.5 h when 0.6 ppm toluene remained is probably most representative of a value that would be obtained in the field with an unlimited supply of substrate. The values of -23.7 and -22.8 at 24 h, long after the target substrate was depleted, most likely have a substantial contribution of CO_2 from basal metabolism of the cells and do not represent values obtained with a constant source of toluene. The -27.0 value does not indicate significant fractionation from the substrate $\delta^{13}C$ (-27.5), indicating that the $\delta^{13}C$ of the original substrate may be predictive of the $\delta^{13}C$ of the resulting CO_2.

Table 2. Degradation of Toluene by *P. mendocina* KR1

Time (hours)	Substrate Concentration (ppm)	Volume CO_2 Generated (cc)	Isotope Ratio for CO_2 (del-C^{13})	Time Zero Adjusted* (del-C^{13})	Background Adjusted** (del-C^{13})
0	25	0.5	-16.5	n.a.	n.a.
0.5	25	0.8	-14.8	-12.0	n.a.
1.25	25	1.2	-13.7	-11.7	n.a.
2	25	1.5	-13.8	-12.5	n.a.
5.5	0.6	3.6	-18.1	-18.4	-27.0
24***	0	3.8	-17.2	-17.3	-23.7
24***	0	4.2	-17.2	-17.3	-22.8
24****	0	3.7	-16.6	-16.6	-22.8
5.5*****	0	1.9	-12.2	-10.7	n.a.

Cells (100 ml @ O.D.550=1.5) were incubated with 25 ppm toluene (iso. ratio -27.5, -27.4)
* Adjusted for 0.5 cc CO_2 with del-C^{13} = -16.5
** Adjusted for 1.9 cc CO_2 with del-C^{13} = -10.7
Ave. Bkg.= 10.7; Iso. Ratio CO_2= 23.1; Fract. Coeff. = 4.4
*** Duplicates; **** Purged with O_2 at 2 hr.; ***** No substrate

Similar results were obtained when *P. mendocina* KR1 degraded JP-4 jet fuel (Table 4). The background corrected value of -24 per mil obtained after 4.5 h of incubation with ~20 ppm JP-4 is close to the original substrate isotope ratio of -25.4. Values later in the incubation may represent contributions from metabolism of other substrates in these batch experiments.

Fractionation of JP-4 and other mixtures is complicated by the fact that the observed ìfractionationî may actually be due to preferential degradation of one or more of the components of the mixture. In order to determine whether the observed isotope ratio of the CO_2 is the result of actual fractionation or preferential degradation, the isotope ratios of all the components of the JP-4 mixture would need to be determined. In addition, the source of the CO_2, i.e. the specific components that have been degraded at any one timepoint, would need to be determined. This would involve analyzing the residual source at every timepoint to determine the amount of each component that has been degraded.

Significant fractionation was apparent only in the values which could not be corrected for background, either because the CO_2 volume was not measured, or the CO_2 volume measured was not significantly above the background volume (measured in no-substrate controls) to allow for this correction (Table 3 and 4).

These batch studies, sampled throughout the degradation of the substrate, demonstrated the $\delta^{13}C$ of the CO_2 changed when degradation was initiated, generally becoming more negative, and then becoming more positive once the substrate was depleted. These values, however, did not represent a constant source of the substrate to the microorganisms. The design of the experiment was modified in the next task to provide a constant source of the substrate(s).

Table 3. Degradation of Chlorobenzene by *P. mendocina* KR1

Time (hours)	Substrate (ppm)	CO_2 (cc)	Isotope Ratio for CO_2 (del-C^{13})	Time Zero Adjusted* (del-C^{13})	Background Adjusted (del-C^{13})
0	16	-	-21.4	n.a.	n.a.
0.5	7.2	-	-21.8	n.a.	n.a.
1	3.1	-	-22.3	n.a.	n.a.
2.5	0	-	-22.8	n.a.	n.a.
5	0	-	NA	n.a.	n.a.
21.2	0	-	NA	n.a.	n.a.
21.2**	0	-	-23.4	n.a.	n.a.

*Not adjusted because CO_2 volume was not measured. **Cells incubated without substrate.
Samples (100 ml @ $O.D._{550}$=1) were incubated with 16 ppm chlorobenzene.
The target compound had an isotope ratio of -27.3 to -27.6.
NA - Not analyzed - all of substrate gone.
N.A. = Not adjusted.

Table 4. Degradation of JP-4 by *P. mendocina* KR1

Time (hours)	Substrate (ppm)	CO_2 Generated (cc)	Isotope Ratio for CO_2 (del-C^{13})	Time Zero Adjusted* (del-C^{13})	Background Adjusted** (del-C^{13})
0	20.6	<0.1	-13.5	n.a.	n.a.
0.5	17.6	0.37	-12.6	-12.3	n.a.
1	14	0.47	-12.8	-12.6	n.a.
2	12.6	0.75	-14.1	-14.2	n.a.
4.5	7.2	1.2	-13.9	-13.9	-24.0
20.5	4.1	2.2	-14.5	-14.5	-17.6
20.5***	2.2	1.9	-12.5	-12.4	-14.5
20.5****	0	1.8	-9.9	-9.7	n.a.
4.5****	0	0.89	-10.5	-10.1	n.a.

Cells (100 ml; $O.D._{550}$=1.5) incubated with 20 ppm JP-4
Original substrate iso. ratio = -25.4
* Adjusted for 0.1 cc CO_2 with del-C^{13} = -13.5
** Not adjusted because CO_2 volume less than background
*** Purged with O_2 at 4.5 h;
**** No substrate added to cells

SECTION IV

Evaluation of the Carbon Isotope Method for its Ability to Differentiate the Source of the CO_2 Resulting from Aerobic Biodegradation of Carbon Sources

Introduction

The purpose of this work was to test the ability of the stable carbon isotope method to determine the source of CO_2 produced during aerobic biodegradation of mixtures of carbon compounds. Carbon isotope ratios obtained with a mixture of substrates were compared to ratios generated from the individual compounds to determine if the method could be used to estimate the progress of bioremediation efforts in the field. Specifically, CO_2 produced from TCE was compared to that produced by several hydrocarbon compounds. These hydrocarbon compounds are often found as co-contaminants with TCE.

Materials and Methods

1. Culture and Growth Conditions

The culture and growth conditions are described in detail in Section III.1.

2. Experimental Procedure

When a sufficient cell density was obtained, the sample was split into several flasks. The flasks contained either one or two hydrocarbon feeders to allow a constant supply of the target substrate in the vapor phase. Hydrocarbon feeders contained toluene, TCE, chlorobenzene, or other components of JP-4 in one feeder and the cosubstrate compound in another. Rubber stoppers wrapped with Parafilm were used to make the flasks air-tight. The flask headspace was then vacuumed and replaced with pure oxygen. The cultures were shaken at 75 rpm and kept at a temperature of 30 C for approximately 24 hours. The following day, 120 cc of headspace gas

was drawn from the flasks and injected into 160 mL sample vials. Prior to the injection of sample, the vials were flushed with pure oxygen and Teflon^{Æ}-sealed.

Results and Discussion

In experiments with a constant source of substrate, $\delta^{13}C$ values adjusted for background CO_2 should be similar to values measured in the field with unlimited substrate. In addition, unadjusted values in experiments where there has been a significant amount of CO_2 generated as a result of the degradation of the substrate, should require minimal correction for background because the majority of the CO_2 will be a result of the degradation. For both *P. mendocina* KR1 and *B. cepacia* PR1, $\delta^{13}C$ values for CO_2 generated as a result of the degradation of toluene were slightly more negative than the starting compound, while values for CO_2 resulting from the degradation of JP-4 were significantly more negative than the starting compound (Tables 5 and 6). These more negative values indicate the CO_2 was enriched in ^{12}C relative to the starting compound. This fractionation for JP-4 was apparent for all of the individual strains tested, as well as the enrichments of toluene degraders from Site 1. The slight fractionation toward a more negative value for toluene was observed for all experiments where a significant amount of CO_2 above background was generated (Table 7).

In a number of these short-term experiments, sufficient CO_2 was not generated over the time period of the incubation to correct for background CO_2. For example, experiments with *P. putida* F1, did not generate CO_2 above the concentration in the no substrate control for any of the compounds tested (Table 8). In these cases, it was impossible to determine whether any fractionation occurred. However, experiments performed with field samples (see Section V) have filled these data gaps.

Table 5. Degradation of Test Substrates by *P. mendocina* KR1

Substrate	Substrate Isotope Ratio (del-C^{13})	CO_2 Generated (cc)	Isotope Ratio for CO_2 (del-C^{13})	Background Adjusted (del-C^{13})
Trichloroethene (TCE)	-31.8	0.2	-18.7	-
Toluene	-27.5, -27.4	4.1	-29.5	-30.0
Toluene + TCE	n.a.	<0.1	-16.9	-
Chlorobenzene (CB)	-27.6, -27.3	0.1	-18.6	-
CB + TCE	n.a.	0.1	-19	-
JP-4	-25.4	2.4	-32.3	-33.4
Methane	-41.2, -41.3	0.2	-18.7	-
No Substrate	n.a.	0.2	-19.9	-

n.a. = not analyzed
"-" means CO_2 may represents background
100 ml cells @ O.D. $_{550}$=1.5

Table 6. Degradation of Target Substrates by Test Organisms

Substrate	Substrate Isotope Ratio (del-C^{13})	CO_2 Generated (cc)	Isotope Ratio for CO_2 (del-C^{13})	Background Adjusted (del-C^{13})
Trichloroethene (TCE)	-31.8	n.a.	n.a.	-
Toluene	-27.5, -27.4	10	-29.2	-29.3
Toluene + TCE	n.a.	<0.1	-21.0	-
Chlorobenzene (CB)	-27.6, -27.3	<0.1	-23.8	-
JP-4	-25.4	1.5	-34.6	-36.3
JP-4 + TCE	n.a.	<0.1	-25.5	-
No Substrate	n.a.	0.2	-23.8	n.a.

100 ml of toluene-grown cells @ O.D.$_{550}$=1
n.a. = not applicable
"-" means CO_2 probably represents background
* background adjusted for 0.2cc with a del-C^{13} of -23.8

Table 7. Degradation of Test Substrates by *P. cepacia* PR1

Substrate	Substrate Isotope Ratio (del-C13)	Isotope Ratio for CO2 (del-C13) / Volume CO2 Generated (cc) Organism						
		P. men. KR1 (O.D.=1.5)	P. put. F1 (O.D.=0.75)	M. vac. JOB5 (O.D.=0.75)	B. cep. PR1 (O.D.=1.0)	B. cep. PR1 (O.D.=1.0)	SITE-1 (O.D.=0.6)	SITE-1 (O.D.=0.75)
Trichloroethene (TCE)	-31.8	-18.7/0.2	-20.0/0.4	-34.7/0.4	-19.3/0.6	n.a.	-22.1/<0.1	-21.7/0.1
Toluene	-27.5, -27.4	-29.5/4.1	-23.8/0.7	-32.8/1.2	-29.5/11.0	-29.2/10.0	n.a.	-24.8/<0.1
Toluene + TCE	n.a.	-16.9/<0.1	-21.7/0.3	-33.4/0.6	n.a.	-21.0/<0.1	n.a.	-22.9<0.1
Chlorobenzene (CB)	-27.6, -27.3	-18.6/0.1	-17.7/0.3	n.a.	-20.0/0.5	-23.8/<0.1	-20.6/<0.1	-23.8/<0.1
CB + TCE	n.a.	-19.0/0.1	-18.4/<0.1	n.a.	-19.1/0.7	n.a.	-21.9/<0.1	n.a.
JP-4	-25.4	-32.3/2.4	n.a.	-37.5/2.1	-32.0/1.9	-34.6/1.5	n.a.	-38.6/1.6
JP-4 + TCE	n.a.	n.a.	n.a.	n.a.	n.a.	-25.5/<0.1	n.a.	n.a.
Methane	-41.2, -41.3	-18.7/0.2	-17.9/0.1	n.a.	-20.0/0.4	n.a.	n.a.	n.a.
No Substrate	n.a.	-19.9/0.2	-15.9/0.5	n.a.	-19.5/0.4	-23.8/0.2	n.a.	-19.3/0.3

n.a. = not analyzed

Table 8. Degradation of Test Substrates by *P. putida* F1

Substrate	Substrate Isotope Ratio (del-C^{13})	CO_2 Generated (cc)	Isotope Ratio for CO_2 (del-C^{13})
Trichloroethene (TCE)	-31.8	0.4	-20
Toluene	-27.5, -27.4	0.7	-23.8
Toluene + TCE	n.a.	0.3	-21.7
Chlorobenzene (CB)	-27.6, -27.3	0.3	-17.7
CB + TCE	n.a.	<0.1	-18.4
Methane	-41.2, -41.3	0.1	-17.9
No Substrate	n.a.	0.5	-15.9

100 ml cells @ $O.D._{550}$=0.75
n.a. = not analyzed

SECTION V

Analysis of Field Samples

Introduction

Samples of both groundwater and soil gas from a contaminated site were analyzed for comparison to data acquired during laboratory studies.

Materials and Methods

The methods used during this study were essentially as previously described. Field samples were collected by Camp, Dresser and McKee field personnel from a site near Denver, CO that was contaminated with chlorinated ethenes and fuels. The existing remediation system was air sparging of the groundwater and a soil vapor extraction system in the vadose zone to physically strip the contaminants from the subsurface. Both soil gas and groundwater samples were collected and analyzed. All vapor sample points were purged prior to taking the sample until the methane, CO_2, and oxygen readings had stabilized.

In addition to standard carbon isotope analyses, individual contaminants within groundwater samples were separated and analyzed for carbon isotope ratios. This work was performed by Professor Teofilo Abrajano and colleagues of the Department of Earth Sciences at Memorial University of New Foundland in St. Johnís, New Foundland Canada.

Analytical Procedure

Samples with both water and soil were separated by filtration and analyzed separately. A 9:1 pentane/dichloromethane extraction (based on method SW846A) was performed for all samples analyzed; analysis by a vacuum headspace procedure was found to be less efficient in extracting VOCs. Samples with low concentrations were concentrated by blowing the solvent down to a smaller volume with N_2; the disadvantage to this procedure was that it precluded analysis of the most volatile contaminants in the water (e.g., benzene).

The gas chromatography/combustion/isotope ratio mass spectrometer system used was a Fisons Isochrom system with a Hewlett Packard 5890II capillary GC and a Fisons Optima high precision isotope ratio mass spectrometer. Analytical precision and accuracy using this instrument are generally better than 0.3 per mil.

Results and Discussion

Results of carbon isotope ratio analysis for the individual contaminants are presented in Table 9. These values do not reflect a large degree of variability for the source compounds in most of the samples. However, in one of the wells sampled (R-03) the m/p-xylene and toluene had a significantly different isotope ratio than the m/p-xylene and toluene from the other sampling points (Table 9). This isotopic difference may indicate the toluene and m/p-xlyene present at R-03 are from a different source than at the other sampling locations, or that fractionation of the compounds has occurred *in situ.* It is possible this toluene and m/p-xylene represents product from a different manufacturer or product produced with different feed stocks. Biotic or abiotic fractionation of substrate compounds prior to degradation has never been documented. Previous studies of biologically mediated fractionation of the source compound have shown residual substrate for multiple carbon molecule contaminants (e.g., toluene, xylene) does not appear to be fractionated during biodegradation (Trust, et al., 1995; Personal communication with Shawn Frape, 1996) although single carbon molecule contaminants (e.g., methane, CO_2) can be highly fractionated (Zyakun, et al., 1979; Zyakun, et al., 1983; Coleman, et al., 1981; Barker and Fritz, 1981; Whiticar and Faber, 1985). No data is currently available to evaluate abiotic fractionation of residual contaminants as they move through the subsurface. A previous study which evaluated the abiotic (i.e., photodegradation, volatilization, sorption) fractionation of residual crude oil found the residual crude oil was not fractionated during or after severe weathering (Winters, et al., 1985). Research on abiotic fractionation of contaminants in groundwater is needed.

Table 9. Summary of Isotope Analysis of Field Groundwater Samples

Compound Analyzed	Isotope Ratio of Compound (del-C^{13})*					
	PW-01 (water)	PW-01 (soil)**	R-54 (water)	R-54 (soil)**	R-03 (water)	MW-107 (water)
Toluene	-25.7	-25.2	n.d.	n.d.	-19.8	n.d.
Ethyl-Benzene	n.d.	n.d.	n.d.	n.d.	-23.5	n.d.
m/p Xylene	-26.8	-27.1	-25.8	-26.4	-23.9	n.d.
o Xylene	n.d.	-27.1	n.d.	n.d.	n.d.	n.d.
Decane	-28.1	-28.0	-27.9	-27.2	n.d.	n.d.
Undecane	-29.7	-28.8	-28.6	-27.6	n.d.	n.d.
PCE	n.d.	-25.8	-26.3	n.d.	n.d.	n.d.
DCB	n.d.	-27.6	-27.6	-27.6	-26.7	n.d.

All carbon isotope ratios are reported in per mil notation with respect to the PDB standard.
Experimental precision using duplicate VOC extraction of the same samples is 0.4 per mil.
* The reported isotope ratios are likely heavier than the true value by 1 to 1.5 per mil.
**Refers to the soil sludge present in the water sample bottles.

The analysis of multiple vapor samples from six different wells at the Denver site indicated little variability in the carbon isotope ratios of the CO_2 collected from the same well (Table 10). Except for samples from well G3, located in an uncontaminated area of the site, these values were similar to those measured for the individual petroleum hydrocarbons (Table 9). This similarity suggests much of the CO_2 collected in soil gas samples was derived from degradation of the hydrocarbons. Some of the variability observed in the field CO_2 measurements between wells is likely due the presence of different mixtures of hydrocarbons in different areas of the site. It may also be a reflection of different sources for the contaminants as seen in the results obtained for the source contaminants in the water samples from well R-03.

Table 10. Summary of Isotope Analysis of Field Vapor Samples (Denver Site)

Sample No.	CO_2 Content (%)	Oxygen Content (%)	Methane Content (%)	CO_2 Isotope Ratio (del-C^{13})	CO_2 Isotope Ratio (del-O^{18})	Background Adjusted* (del-C^{13})	Notes
VP4-001	10.4	3.2	2.4	-25.76	21.48	-25.94	Replicate vapor samples from each well
VP4-002	-	-	-	-25.77	21.56	-25.95	
VP4-003	-	-	-	-25.79	21.54	-25.97	
VP4-004	-	-	-	-25.78	21.20	-25.96	
VP4-005	-	-	-	-25.76	21.10	-25.94	
VP4-006	-	-	-	-25.72	21.22	-25.90	
VP7-001	10.5	0.3	2	-25.12	19.65	-25.28	
VP7-002	-	-	-	-25.16	19.60	-25.32	Replicate vapor samples from each well
VP7-003	-	-	-	-25.05	19.84	-25.21	
VP7-004	-	-	-	-25.03	19.89	-25.19	
F2-001	2.9	16.6	0.6	-26.95	20.40	-27.79	
F2-002	-	-	-	-26.91	20.53	-27.74	Replicate vapor samples from each well
F2-003	-	-	-	-26.92	20.47	-27.75	
F2-004	-	-	-	-26.94	20.35	-27.78	
F3-001	2.9	16.3	0.2	-27.15	20.31	-28.01	
F3-002	-	-	-	-27.14	20.29	-28.00	Replicate vapor samples from each well
F3-003	-	-	-	-27.10	20.36	-27.95	
F3-004	-	-	-	-27.14	20.34	-28.00	
F3-005	-	-	-	-27.12	20.36	-27.98	
ORZD-001	11.1	9.2	0	-23.62	18.24	-23.73	
ORZD-002	-	-	-	-23.62	18.26	-23.73	Replicate vapor samples from each well
ORZD-003	-	-	-	-23.53	18.35	-23.64	
ORZD-004	-	-	-	-23.54	18.37	-23.65	
G3-001	0.3	20.8	0.2	-18.44	-	n.a.	
G3-002	-	-	-	-20.98	-	n.a.	Replicate vapor samples from each well

All carbon isotope ratios are reported in per mil notation with respect to the PDB standard.
Experimental precision using duplicate VOC extraction of the same samples is 0.4 per mil or better
* Adjusted for 0.3% CO_2 with del-C^{13} = average background (i.e., -19.7).

To confirm laboratory results were comparable to those derived from analysis of field vapor samples, groundwater samples were added to BSM and incubated in the laboratory without addition of microorganisms or substrates. Degradation of the substrates within the sample resulted in the production of CO_2 with isotope ratios between -18.6 and -25.7. The two water samples which had a vapor sample taken at a nearby point in the field showed similar $\delta^{13}C$ values for the CO_2 evolved during the laboratory incubation (Table 11). Ideally, for this type of comparison, the vapor samples and the water samples would be taken from the same sampling location.

Table 11. Comparison of Field and Laboratory Data

Sample Location	Nearby Vapor Sampling Point	Average Field CO_2 Isotope Ratio (del-C^{13})	Laboratory CO_2 Isotope Ratio (del-C^{13})
PW-01	ORZD	-23.58	-21.00
R-54	n.a.	-	-18.60
R-03	VP-7	-25.09	-25.70
MW-107	n.a.	-	-19.90
Background	G-3	-19.71	-

n.a. = nearby vapor sampling point not available.

To further assess the isotope ratios of CO_2 produced during degradation of site contaminants, water samples from the Denver site were incubated with toluene in the headspace to enrich for toluene-degrading microbes. The samples were then amended with target constituents and incubated in the laboratory. As reported in Table 12, CO_2 produced during degradation of toluene had the same relative range of isotope ratios as those observed in CO_2 produced during toluene degradation by individual toluene-degrading microbes (Table 7). Toluene degradation with enrichments in Denver site water (Table 12) again resulted in only the slight to non-existent fractionation observed in pure culture studies (Table 7). CO_2 produced during incubation of the site samples with TCE had an isotope ratio of -29.6 per mil (Table 12), which is more positive than the $\delta^{13}C$ value of the source compound. The amount of CO_2 generated in this TCE degradation experiment (9.7 cc) is sufficient to give a value which would most likely not require significant correction for background. Therefore, this fractionation, with the CO_2 resulting from degradation being enriched in ^{13}C relative to the source, is probably predictive of what field samples would show if the sole contaminant was TCE. As expected, a combination of toluene and TCE had a $\delta^{13}C$ value in between those for TCE only and toluene only. As observed previously in both pure culture studies and in studies with bacterial enrichment cultures (Table 7), JP-4 degradation in site samples resulted in $\delta^{13}C$ values for CO_2 significantly fractionated

from the source compound. The value for the isotope ratio in the CO_2 is significantly more negative or lighter than the source compound $\delta^{13}C$. This indicates the resulting CO_2 is enriched in ^{12}C relative to the JP-4.

Table 12. Degradation of Test Substrates in Denver Site Water

Substrate Added	Isotope Ratio Substrate (del-C^{13})	CO_2 Generated (cc)	Background Adjusted (del-C^{13})
TCE	-31.8	9.7	-29.6
Toluene	-27.5, -27.4	>10	-27.7
Toluene + TCE	n.a.	0.9	-28.1
JP-4	-25.4	6.9	-31.5

100 ml of toluene-grown cells from site water @ $O.D._{550}$=1
n.a. = not analyzed

An exciting finding derived from the incubation of site samples with mixed substrates was that the carbon isotope ratio of CO_2 produced during degradation of a mixture of TCE and toluene was between the ratios observed when individual substrates were degraded (Table 12). The even larger difference between the $\delta^{13}C$ values for the CO_2 from JP-4 and TCE degradation suggests the degradation of one or both of these compounds when both are present should be demonstrable by SCIRA. The results of experiments with enrichments of indigenous bacterial populations from the Denver site are very similar to the results observed with individual organisms, and they confirm SCIRA might allow one to identify when TCE and petroleum hydrocarbons cosubstrates are being degraded simultaneously.

SECTION VI

RECOMMENDATIONS

The goal of this project was to develop a laboratory system to be used in conjunction with field samples to determine whether both contaminants (cosubstrate and chlorinated solvent) at a site are being degraded. Initially, this system would concentrate on TCE and JP-4, but would eventually include other contaminant mixtures and more than two contaminants. The systems would eventually develop a sufficient database so laboratory studies will not be necessary. Determination of degradation could be made solely on the basis of a limited number of soil vapor and/or groundwater samples.

The microcosm system originally designed for this study did not adequately reflect carbon isotopes that would be obtained in a field system. The development of the ìconstant feedî microcosms enabled us to obtain values which would move closely reflect field values, however, lack of access to an appropriate site for this study did not allow us to confirm the validity of this method.

The following includes both recommendations for future research on the use of SCIRA for analysis of biodegradation and work proposed for Phase II of this project. In future work, soil microcosm studies performed in the laboratory should parallel the experiments to be performed in the field. These microcosms can be used to follow the kinetics of degradation of the hydrocarbons and chlorinated ethenes under the different venting conditions employed in the field. These experiments should include the effects on fractionation of toluene and TCE, and JP-4 and TCE in clean soils collected from the site.

Laboratory bench-scale studies can be conducted on soil samples collected in the field during test plot construction. The tests should evaluate, under ideal conditions, system mass balance, stable isotope fractionation and biodegradation kinetics for air only and oxygen only in the venting gas. Flow rates can be adjusted to minimize any physical removal of contaminants from the

microcosms. The primary objective of the microcosm studies is to develop a laboratory method for reliably predicting site specific field fractionation values to eliminate the need for a detailed field study. This laboratory protocol could be used to validate results of a simple and inexpensive field sampling protocol.

A field test should be performed in a field-scale ëclosedí plot in order to demonstrate SCIRA in a well-defined system in a more realistic setting. The field test should include a bioventing system installed at a site contaminated by TCE and JP-4 where the vadose zone can be isolated. Bioventing with both air and pure oxygen would allow one to determine if any isotope effects occur due to elevated oxygen levels. Laboratory microcosm studies performed with site materials would determine whether laboratory systems can be developed to adequately predict field results.

In addition to stable carbon isotopes in soil gas samples, analysis of stable chlorine isotopes in groundwater samples may provide additional evidence for degradation of chlorinated ethenes and help to close the mass balance on the degradation without extensive sampling. The analysis of chlorine isotopes in environmental samples is not currently commercially available due to a lack of significant research in this area.

It seems unlikely that isotope fractionation related to biological degradation is site specific, however, this hypothesis is based on limited amount of data. With a sufficient database of $\delta^{13}C$ values for CO_2 evolved as a result of degradation of chlorinated ethenes and petroleum hydrocarbons, it would be possible to rely solely on field data as a means to demonstrate degradation of one or more compounds with an inexpensive analysis of stable isotope ratios.

Comparing the results obtained in well-controlled field and laboratory systems to stable isotope analysis at other bioventing sites will assist in the development of a protocol for monitoring bioventing and biosparging sites. These protocols will enable the user to demonstrate degradation of both cosubstrate and chlorinated ethenes with inexpensive soil gas and groundwater sampling. These protocols may also provide users with parameters which can be used to monitor and optimize bioventing/biosparging processes and enable efficient termination of these systems at scientifically determined endpoints.

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